Hemodynamic Responses and c-Fos Changes Associated with Hypotensive Hemorrhage:

Standardizing a Protocol for Severe Hemorrhage in the Conscious Rat

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ABSTRACT

The central mechanisms underlying the transition from compensation to decompensation during severe hemorrhage (HEM) are poorly understood. Furthermore, a lack of consistency in HEM protocols exists in the current literature. This study assessed the cardiovascular response and Fos-like immunoreactivity (FLI) in specific brain regions following severe HEM at three rates of HEM (2, 1, or 0.5 ml/kg/min) in conscious rats. Heart rate (HR) and arterial pressure were recorded during the withdrawal of 30% of total blood volume (TBV). Data from animals hemorrhaged at the fast (F-HEM, n = 6), intermediate (I-HEM, n = 7), or slow (S-HEM, n = 7) rates were compared to saline (SAL, n = 5) and hypotensive (hydrazaline-induced, HYDRAZ, n = 5) controls. All HEM rates produced similar degrees of hypotension at the time of 30% TBV withdrawal. All HEM rates also produced bradycardia, but the change in HR was only significant in the F-HEM and I-HEM groups. Associated with I-HEM and F-HEM, but not HYDRAZ treatment, were significant increases in FLI in the caudal ventrolateral periaqueductal gray (PAG), the central lateral nucleus of the rostral parabrachial nucleus, and locus coeruleus compared to SAL treatment. I-HEM also induced significant increases in FLI in the dorsomedial PAG, A7 region, and the cuneiform nucleus compared to SAL. S-HEM did not induce any significant change in FLI. Our results suggest that hemorrhage at a rate of 1 ml/kg/min may be most useful for investigating the potential role of the rostral brainstem regions in mediating hemorrhagic decompensation in conscious rats.
INTRODUCTION

Conscious mammals subjected to severe, progressive hemorrhage have been shown to pass through two hemodynamically distinct phases. Upon initial blood loss, an augmented sympathetic drive results in an elevated heart rate (HR) and increased peripheral resistance. In this compensatory phase, the declining cardiac output is not large enough to cause a fall in mean arterial pressure (MAP). When blood loss approaches 25-30% of total blood volume, a decompensatory phase consisting of sympathetic withdrawal, hypotension, and bradycardia ensues. Anesthesia can significantly alter the response pattern to blood loss (21, 44). Anesthetized animals, in general, show little if any compensatory response to hemorrhage and therefore enter the decompensatory phase earlier than conscious animals following loss of much smaller volumes of blood. Additionally, under anesthesia, MAP drops more quickly and is typically proportional to the amount of blood withdrawn (21). The duration of the decompensatory phase is often associated with increased peripheral organ damage and a reduced chance of survival (36).

Over the last ten years there has been increased interest in understanding the central-neural mechanisms underlying hemorrhage-induced sympathoinhibition (HIS) with the hope of identifying therapies that might delay the onset of the decompensatory response and facilitate recovery. While the mechanisms underlying the initial, compensatory phase of hemorrhage are fairly well accepted (baroreceptor unloading) (9, 14, 36), the signal that prompts the transition to a decompensatory state is less clear. There is some evidence, however, that input to the midbrain is critical for inducing HIS (13). For example, blockade of the ventrolateral periaqueductal gray (VLPAG) has been shown to both delay and attenuate hemorrhage-evoked hypotension (7). Furthermore, in a recent brain transection study, it was demonstrated that pre-trigeminal
decerebrate rat, lacking midbrain-brainstem communication, had a markedly attenuated HIS response to severe hemorrhage compared to controls (42). In contrast, pre-collicular decerebrate animals (in which midbrain-brainstem connections were still present) displayed a prolonged decompensation compared to controls. These findings support the current idea that midbrain structures play a pivotal role in the onset of HIS. Interestingly, in the same study it was shown the prolongation of the decompensatory response observed following pre-collicular decerebration was only observed in animals that underwent a 30% hemorrhage over 20 minutes but was not present when 30% hemorrhage occurred over 40 minutes. Thus, the rate of hemorrhage may profoundly impact brain mechanism(s) contributing to or perhaps initiating HIS.

To our knowledge, no previous studies have combined a thorough evaluation of the cardiovascular outcome of constant-volume hemorrhage over different withdrawal times with the identification of the associated specific CNS regional sites of activation. Furthermore, an in-depth evaluation of the impact of rate of hemorrhage on the brain mechanisms involved in HIS might be considered necessary at the present time, since a review of the literature reveals a distinct lack of consistency in hemorrhage protocols between studies (5, 11, 21, 29, 37, 40, 41).

The purpose of this project was to assess the neural responses to different rates of hemorrhage in order to develop a standard protocol that best demonstrates HIS during severe hemorrhage in conscious rats for use in future studies. Because specific regions throughout the CNS have been shown to play integral roles in regulation of homeostasis during hemorrhage (5, 7, 20, 42), we felt that, in addition to hemodynamics, evaluating neural activity in response to hemorrhagic hypotension would offer a more complete picture of the natural consequences resulting from severe blood loss. Accordingly, this study aimed to evaluate hemodynamic
responses and c-Fos immunoreactivity in specific regions of the brain following different rates of severe (30% total blood volume, TBV) hemorrhage in the conscious rat. Two areas of particular interest were the VLPAG and the lateral parabrachial nucleus (LPBN) because of the independent and integrated roles they have been shown to play in response to cardiovascular challenge (3, 7, 10, 24, 35, 45). We hypothesized that the fastest rate of hemorrhage would induce the earliest and greatest level of decompensation and that this would be associated with increased levels of c-Fos immunoreactivity in the VLPAG and LPBN.

METHODS

General Preparation. All experimental procedures were approved by the Animal Care and Use Committee at the University of Florida. Male Sprague-Dawley rats (357±6 g, Harlan Industries, Minneapolis, IN) were anesthetized with an i.p. injection of ketamine/xylazine/acepromazine (80-100/8-20/1-3 mg/kg, respectively) and then randomly placed into one of three groups: hemorrhage (HEM); saline volume control (SAL); or hydralazine pressure control (HYDRAZ).

Following assignment to a group, all rats were surgically instrumented with catheters (PE-10 connected to PE-50 tubing, Braintree Scientific, Braintree, MA) filled with heparinized saline (100 IU/ml). HEM rats were instrumented with two femoral arterial catheters. SAL and HYDRAZ rats were instrumented with a femoral venous and a femoral arterial catheter. Catheters were then tunneled subcutaneously, exteriorized at the nape of the neck, and sealed with 23-gauge obturators until the day of the experiment. Analgesics (Rimadyl, 0.01 ml/kg; Buprenorphine, 0.01 ml/kg) were administered subcutaneously following catheterization and animals were allowed 48 hours to recover. During recovery, animals were housed singly under
controlled illumination (12-hour cycle) with food and water ad libitum. The day following catheter placement, animals were brought to the lab to ensure catheter patency and for acclimating purposes. Animals were weighed, lightly handled, and allowed to sit quietly for 2-3 hours in the testing chamber (9x9 inch bucket) they would be placed in for the experiment. Animals were returned to their home cages following acclimation. Animals that lost more than 10% of their body weight over two days following surgery were excluded from the study.

**Experimental Protocol.** On the day of the experiment, animals were brought to the lab, weighed, placed in the testing chamber and a single arterial catheter was connected to a calibrated pressure transducer in-series with an amplifier (Stoelting, Wooddale, IL). The arterial and venous catheters were then attached to a swivel system (Instech, Plymouth Meeting, PA) so the animals could move unrestrained about the testing chamber. Both pulsatile and mean arterial pressures (MAP) were recorded on-line at 100 Hz using a Cambridge Electronics Design computer interface and Spike2 data software. HR was derived on-line from the interval between peak systolic pressure waves in the arterial pressure (AP) trace.

After 60-90 minutes of quiet rest, the experiment began. First, baseline AP, MAP, and HR were recorded for a 30-minute baseline period. Next, animals underwent one of 5 experimental procedures: 1) slow hemorrhage (S-HEM, 0.5 ml/kg/min, n = 7); 2) intermediate hemorrhage (I-HEM, 1.0 ml/kg/min, n = 7); 3) fast hemorrhage (F-HEM, 2.0 ml/kg/min, n = 6); 4) saline control (SAL n = 5); or 5) hydralazine control (HYDRAZ, n = 5). All hemorrhaged animals underwent a 30% blood volume extraction through the second arterial catheter. Total blood volume (TBV) was calculated using a previously reported equation for estimation of rat blood volume: 

\[(0.06 \text{ ml/g}) \times \text{(body weight in g)} + (0.77)\] (26). S-HEM, I-HEM, and F-HEM were performed over 40, 20, and 10 minutes, respectively. For HYDRAZ animals, 1 ml of
hydralazine (3mg/kg) was infused over 60-seconds through the venous catheter in order to induce a level of hypotension similar to that induced by HEM (15, 34). For the SAL animals, 1 ml of heparinized saline (0.9% NaCl, 2 IU/ml) was infused over 60-seconds through the venous catheter.

Ninety minutes after the cessation of hemorrhage or drug infusion protocols, animals were administered a lethal dose of sodium pentobarbital (100-150 mg/kg) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde 90-minutes. Brains were removed and post-fixed in 4% paraformaldehyde for 24 hours followed by 24-48 hours of immersion in cryoprotectant solution (30% sucrose) prior to cryostat sectioning.

_Fos Immunocytochemistry._ Extracted brains were cut into 40 micrometer coronal sections and processed for Fos-like immunoreactivity (FLI) as previously described (18). Briefly, free floating sections were washed in sodium phosphate buffered saline (PBS, pH 7.4) followed by a second wash in a 3% goat serum-PBS-triton X100 solution (3% GS-PBS-TX) to prevent nonspecific binding. Sections were then incubated for 24 hours in rabbit anti-c-Fos primary antibody (1:2000 dilution, sc-52r, Santa Cruz Biotechnology). Following another wash in 1% GS-PBS-TX, sections were incubated in goat anti-rabbit biotin (Jackson ImmunoResearch Laboratories, Inc., 111-065-144) for two hours and re-washed (1% GS-PBS-TX) prior to being placed in avidin-biotin peroxidase complex (ABC Vectastain Kit, Vector, Burlingame, CA). Sections were put through a final wash (1% GS-PBS-TX) followed by visualization of the FLI with a chromagen solution (0.05% diaminobenzidine hydrochloride [DAB], 2.5% ammonium sulfate, 0.033% hydrogen peroxide in 0.05 M Tris-HCl, Vector). Sections were then mounted onto glass slides, air-dried, dehydrated in a graded alcohol and CitriSolv (Fisher Scientific) series, and coverslipped.
Neuroanatomical Quantification of FLI. For each animal, two representative sections from each brain area of interest were imaged (Axioskop, Carl Zeiss; 5X) and analyzed for the number of FLI neurons present by a technician blinded to the experimental conditions. The software used for FLI quantification (Metamorph) allows the investigator to assign color, object size (7-10 µm), and/or density ranges specific to Fos-positive cells, as determined by the investigator. Once these ranges have been preset, the software is then able to “recognize” and record FLI in a specified field of the image. This allows greater consistency and decreased human error in quantifying FLI between images.

Figure 1 shows a schematic of the rostral, middle, and caudal coronal sections of the LPBN and PAG analyzed in this study. The criteria used for selecting specific sections of the LPBN included the shape of the superior cerebellar peduncle, the width of the LPBN from the superior peduncle to the ventral spinocerebellar column, and the width of the ventral spinocerebellar tract. The criteria for choosing specific PAG sections were based on the shape of the central aqueduct, the shape and width of the dorsal and ventrolateral columns, and the presence of the oculomotor nucleus. Other areas imaged and quantified for FLI included the locus coeruleus (LC, interaural -0.68 to -0.80), the cuneiform nucleus (CnF, interaural 0.48 to 0.60), Kolliker-Fuse (KF) and A7 (imaged in the same section and at the level of the rostral LPBN) (33). Several standardized “masks” were prepared for each level of each brain nucleus using counterstained brain sections as well as guidance from previously diagramed images of the brain areas of interest (2, 19, 25). These masks were superimposed over corresponding images using Adobe Photoshop 7.0 in order to outline boundaries of selected brain areas and the different subnuclei within the PAG (2) and the LPBN (24) prior to FLI analysis. Masks were prepared in such a way as to allow fitting to individual brain dimensions as well as various
angles of cut, but to still maintain the integrity of the approximate shapes and proportions of relative subnuclei.

Cardiovascular Measurements. MAP and HR were averaged over five-minute intervals for each experiment. MAP and HR values from 5 minutes prior to the onset of HEM or drug were averaged to give a single baseline value (0 min.). Following the onset of HEM or drug infusion, the first two 5 minute averages (5 min. and 10 min.) and then every other 5 minute average (20 min., 30 min. etc.) were used for calculation of group averages.

Statistical Analysis. A one-way ANOVA was used to determine if there were any significant differences in baseline MAP or HR between treatment groups. A two-way ANOVA with repeated measures was used to identify the effects of treatment (i.e. HEM or HYDRAZ) on MAP and HR over time (minutes 0, 5, 10, 20, 30, 40, 50 and 60). When indicated, paired or unpaired Bonferroni t-tests were then used to isolate differences relative to baseline (minute 0) within treatment groups or between treatment groups at specific time points. The accepted P value ($P<0.05$) was adjusted for the number of t-tests performed ($n = 7, P<0.007$). To determine whether MAP or HR at the offset of HEM within each treatment group were significantly different from the HYDRAZ treatment group at the same time point, an unpaired t-test was used. Differences were considered significant when $P<0.05$.

FLI data from all regions except the LPBN were analyzed using a one-way ANOVA comparing the effect of treatment (i.e. HEM or HYDRAZ) on FLI levels within each specific rostral-caudal section chosen for analysis. In the LPBN, FLI data were analyzed using a two-way ANOVA comparing the effect of treatment and subnuclei on FLI levels within each specific rostral-caudal section. If a significant effect was indicated, unpaired Bonferroni t-tests were used to reveal differences between SAL versus other treatments on FLI levels with each brain region.
The accepted P value ($P<0.05$) was adjusted for the number of t-tests performed ($n = 4$, $P<0.012$). All data are presented as mean ± SEM.

RESULTS

*Cardiovascular response to hypotension versus severe hemorrhage.* Baseline MAP and HR for all groups of animals is shown in Table 1. There was no significant difference in resting MAP and HR between groups at the start of the experiments. Figures 2 & 3 illustrate the average change in MAP and HR over time for all groups following treatment. For SAL animals, there was no change in MAP or HR from baseline throughout the experiment (Fig. 2). HYDRAZ animals, on the other hand, showed a significant decrease in MAP from baseline starting at 5 min. following HYDRAZ administration and continued throughout the experiment (Fig. 2A). Additionally, at 20 minutes and for the remainder of the measurement period, the MAP of HYDRAZ-treated animals was significantly different from SAL animals. In response to the HYDRAZ-induced hypotension, HR increased significantly above both baseline and the HR of SAL animals at 10 minutes post-injection and remained elevated throughout the experiment (Fig. 2B).

HEM also induced a persistent hypotension, but in all groups, MAP did not drop significantly below baseline until > 15% TBV had been withdrawn (Fig 3A). In the S-HEM group at min. 30, when approximately 23% of the TBV had been withdrawn, MAP fell significantly below baseline (Fig. 3A). In both the I-HEM and F-HEM groups, MAP was identified to be significantly below baseline at 20 and 10 min. post HEM onset, respectively. In all three HEM groups, the lowest MAP was recorded at the offset of HEM. MAPs measured at the offset of HEM or the time of 30% TBV withdrawal was not significantly different between
HEM groups. Furthermore, a comparison of MAP at the time of 30% TBV withdrawal in all HEM groups versus HYDRAZ at a similar time point (Table 2) demonstrated that for both S-HEM and I-HEM, the decrease in MAP at the time of HEM completion was similar to that induced by HYDRAZ. In contrast, at corresponding time points, the decrease in MAP for the F-HEM group was significantly different from the HYDRAZ group.

HR showed an increase from baseline during the first half of the HEM protocol for all groups. However, the peak increase in HR was only significantly different from baseline (Fig. 3B) in the I-HEM group during this initial compensatory period. In all HEM groups the peak drop in HR occurred at the offset of HEM. However, because of a large amount of inter-individual variability, the decrease in HR for the S-HEM group was not significantly different from baseline at any time point following the onset of HEM. Furthermore, the decrease in HR for both the F-HEM and I-HEM groups was only significantly different from baseline at 20 and 30 minutes following the onset of HEM, respectively (Fig. 3B). The HR of the F-HEM group was also significantly different from baseline at 30 min. following HEM onset. HR in the I-HEM group was significantly different from baseline at 50 and 60 minutes following the onset of HEM. HR values recorded at the offset of the HEM when 30% of TBV had been withdrawn were not significantly different between HEM groups. However, HR values at the offset of HEM for all groups, at the time of peak hypotension, were significantly lower than HYDRAZ values at the same time points (Table 2).

**FLI in brainstem nuclei following severe hemorrhage.** Figure 4 shows representative middle and caudal sections of the PAG for visual comparison of FLI labeling in a SAL and an I-HEM rat. Regions of the PAG chosen for quantification of FLI included both the dorsomedial PAG (DMPAG) and the ventrolateral PAG (VLPAG), based on their known physiological
contributions to sympathoexcitation and sympathoinhibition, respectively (7, 17). As shown in Fig. 4, I-HEM induced increased levels of FLI, relative to SAL, throughout the dorsal and ventral PAG. FLI labeling for the SAL group depicts basal levels of neural activation in these conscious animals, as MAP and HR were unchanged for the duration of the experiment. Accordingly, all changes in FLI following HEM or HYDRAZ were compared to FLI levels in the SAL treated animals.

Figure 5 illustrates average FLI levels in the PAG for all treatment groups. In general, both HEM and HYDRAZ treatment induced large changes in FLI in the VLPAG compared to SAL treatment and there was a significant effect of treatment ($P<0.001$) in both the caudal and middle VLPAG. In the caudal VLPAG, however, only I-HEM and F-HEM induced a significant increase in FLI compared to SAL. In the middle VLPAG, all HEM rates and HYDRAZ induced a significant increase in FLI above SAL. In contrast, in the caudal DMPAG there was no significant effect of treatment on FLI. In the middle DMPAG there was a treatment effect ($P<0.002$), but only I-HEM induced a significant increase in FLI compared to SAL.

Figure 6 shows representative rostral, middle, and caudal sections of the LPBN from a SAL-treated versus an I-HEM and an F-HEM rat. The top three panels show increased levels of FLI in the central subnucleus of the rostral LPBN in both HEM groups compared to SAL. In the middle and caudal LPBN, increases in FLI in the HEM above SAL treated animals were primarily located in the external and dorsal subnuclei.

The average increase in FLI induced within subnuclei of the rostral, middle and caudal LPBN following HEM and HYDRAZ is shown in Figure 7. In the rostral LPBN, a main effect of treatment ($P<0.0004$) and subnuclei ($P<0.0001$), as well as an interaction between factors ($P<0.008$), was observed. Comparisons within the individual subnuclei demonstrated that
within the central subnucleus of the rostral LPBN both I-HEM and F-HEM increased FLI significantly above SAL-induced levels. In contrast, in the superior lateral subnucleus of the rostral LPBN, I-HEM as well as HYDRAZ treatment induced a significant increase in FLI above SAL treatment.

In the middle and caudal LPBN, there was a significant effect of subnuclei (P<0.001 for both) and treatment (middle: P<0.0001; caudal: P<0.02), but no interaction between these two factors (middle: P<0.4; caudal: P<0.8). Thus, a comparison of significant effects of treatment on FLI within individual subnuclei was not permitted. In the middle LPBN however, irrespective of individual subnuclei within the middle LPBN (Fig. 7B), all HEM and HYDRAZ treatments induced increased levels of FLI relative to SAL. In the caudal LPBN, only F-HEM and HYDRAZ showed significantly more FLI than SAL (combined subnuclei).

Figure 8 shows the average increase in FLI across treatment groups for four other rostral brainstem regions also quantified, including the CnF, LC, KF (a subnucleus of the PBN), and A7. In all four regions, there was a significant effect of treatment on FLI (P<0.01). In both the CnF and A7 nuclei, only I-HEM induced a significant increase in FLI compared to SAL. In the LC (Fig. 8B), both I-HEM and F-HEM, but not HYDRAZ, induced a significant increase in FLI labeling compared to SAL. In contrast, both HYDRAZ and I-HEM induced significant increases in FLI compared to SAL treatment in KF (Figs. 8B & 8C, respectively).
DISCUSSION

It is generally accepted that the amount of blood loss necessary to induce hypovolemic decompensation in a rat is between 15-30% of the animal’s TBV (38). However, the rate at which this volume is lost may impact the transition from compensation to decompensation and, thus, brain mechanism(s) recruited to meet the physiological challenge (42). In the present study, all rates of HEM induced clear compensatory and decompensatory stages. In all instances, during blood loss of up to 15%, there was a compensatory tachycardia, and MAP was well maintained. Yet, only during I-HEM was the increase in HR during the compensatory phase significantly different from baseline. Following 30% TBV withdrawal, all hemorrhaged animals had MAPs that were significantly reduced from baseline. Both F-HEM and I-HEM groups showed a corresponding drop in HR that was significantly different from baseline at 10 minutes following the offset of HEM. Furthermore, in the I-HEM group a significant reduction in HR was observed between 30 and 40 min. following the offset of HEM. These observations demonstrate that there are marked differences in autonomic regulation of MAP and HR when severe hemorrhage occurs at different rates of blood loss.

Examination of FLI in the rostral brainstem identified several regions that might be selectively involved in autonomic regulation during severe hemorrhage. These regions showed significant increases in FLI following I-HEM but not in response to HYDRAZ treatment; including the middle DMPAG, the caudal VLPAG, the central lateral subnucleus of the rostral LPBN, LC, A7, and the cuneiform nucleus. Other brain regions examined demonstrated increased levels of FLI associated with both HEM and HYDRAZ compared to SAL controls. This suggests that activation of these specific brainstem sites was more closely related to autonomic regulation in response to hypotension than the hypovolemia and HEM-associated
adjustments autonomic regulation. Interestingly, the outcome of our study did not support our original hypothesis that F-HEM would induce the greatest change in both cardiovascular and FLI. In contrast, our results suggest that utilization of a hemorrhage at an intermediate rate of 1 ml/kg/min for 30% TBV hemorrhage may be most useful for investigating the potential role of the rostral brainstem regions in mediating hemorrhagic decompensation in conscious rats.

**Methodological considerations.** Several methodological factors must be considered when interpreting the results of the present study. First, in the present study we chose to use HYDRAZ for our non-volume depleted hypotensive controls because of its use in previous studies (15, 34), as well as its known and reliable hypotensive actions. However, HYDRAZ proved to be a potent and long lasting vasodilator. The induction of such a long lasting hypotension may have added additional stress to the animals. In retrospect, a shorter acting vasodilator, such as sodium nitroprusside, may have been a more suitable control and may have better mimicked the response seen with our selected method of hemorrhage.

The second factor to consider in this study is the use of FLI to identify specific regions of the brain involved in cardiovascular control when multiple stimuli and physiological changes occur over a relatively short time period. FLI is induced following neuronal excitation and depolarization and is an indicator of changes in neuronal activation associated with a stimulus. However, the resolution of these changes to specific time points is limited. In our study, all animals were sacrificed at 90 minutes following the offset of hemorrhage. Accordingly there should have been a good correlation between FLI levels and the maximum drop in MAP (8). However, during severe hemorrhage many other physiological changes occur in an attempt to survive the insult (4, 32). As a result, we can only correlate changes in FLI with the recorded
cardiovascular changes. Further studies are needed to more definitively identify the role of each region identified in the present study in mediating hemorrhagic decompensation or recovery.

**Cardiovascular response to hemorrhage.** The only other study that we are aware of that has previously addressed the issue of hemorrhage rate maintaining the volume withdrawn constant was by Troy and colleagues (42). In that study, 30% TBV hemorrhages were performed in conscious rats over 20 or 40 minutes. Blood pressure responses were similar to those reported in the present study. That is, MAP was well maintained for the first 15% of TBV loss; but by the time 30% TBV had been withdrawn there was approximately a 50% drop in MAP irrespective of hemorrhage rate. The HR responses, however, were considerably different. In their study, the faster rate of hemorrhage, which corresponded to our intermediate rate (I-HEM), produced a reflexive tachycardia that peaked at ~38% above baseline following 30% hemorrhage, and HR remained elevated compared to the pre-hemorrhage baseline during recovery. The slower rate of hemorrhage (30% TBV loss over 40 minutes corresponding to our S-HEM group) also produced a tachycardia in response to hemorrhage, but it was much more variable and reached a peak of only ~10% above baseline. In contrast, we reported a transient tachycardia in both the S-HEM and I-HEM groups, which was only ~8-15% above baseline following 15% TBV loss. As blood loss continued, HR began to drop below baseline the peak drop in HR occurred at or just following 30% total blood loss and persisted throughout recovery. The discrepancy between studies might be explained by differences in protocol. For example, animals in our study were allowed 48 hours to recover from surgery, while the rats in the previous study were hemorrhaged between 70 and 120 minutes following catheterization, in which inhalant halothane anesthesia was used. Since halothane can remain in the body’s tissues for at least two hours following anesthetic levels of exposure to it (12), remnant anesthesia may
have modified the HR response to hemorrhage in their study. Similarities between our study and other hemorrhage studies with longer post-surgery recovery times suggest that anesthesia, even 4 to 6 hours following withdrawal (3, 7, 40), can markedly alter autonomic control of HR (27) and presumably brain mechanisms recruited for autonomic adjustments during hemorrhage.

**Pattern of FLI in the rostral brainstem following hemorrhage.** Brain regions previously identified to be responsible for the switch from a compensatory response to decompensation during hemorrhage have been isolated to the rostral brainstem (13). More specifically, both the VLPAG in the midbrain (7) and the LPBN in the rostral pons (3) have been shown to play important roles in hemorrhagic decompensation. In the present study, increased levels of FLI were observed in both the VLPAG and LPBN in response to HEM compared to control (SAL). However, only in the caudal VLPAG and the rostral LPBN were the HEM-induced changes in FLI distinguished from changes in FLI induced by HYDRAZ or hypotension alone. This raises the possibility that neurons in these regions are critical in mediating autonomic responses associated with hemorrhagic decompensation.

In the present study increases in FLI were consistently observed in the VLPAG in response to all three rates of HEM. This observation supports physiological data from Cavun and Millington (7) demonstrating that synaptic blockade in the VLPAG markedly attenuates both the hemorrhagic hypotension and hemorrhage-induced changes in HR. Furthermore, in a recent study by Schadt and colleagues (39), neurons in the VLPAG were shown to display discharge patterns indicative of mediating HIS. Our observation that HEM induced a significant increase in FLI in the VLPAG also corroborates the results of a previous study investigating FLI following HEM (22). However, because hypotension alone can also induce FLI in the VLPAG (28, 30), in the present study we also evaluated the effect of hypotension (HYDRAZ) on FLI in
the VLPAG. Our results identified that, similar to HEM, HYDRAZ induced a significant increase in FLI in the middle VLPAG compared to control. In the caudal VLPAG however, the response to HYDRAZ was more variable and was consequently not significant. Yet, increases in FLI in the caudal VLPAG in response to both I-HEM and F-HEM were significant. This raises the possibility that neurons in the middle VLPAG may be more important in regulating autonomic responses to hypotension, whereas caudal VLPAG neurons may be more involved in regulating cardiovascular function during severe HEM. This observation is supported by data demonstrating that both the neuroanatomical connectivity (2) and control over different vascular beds of the middle vs. caudal VLPAG are distinctive (6).

In the present study we also observed a small but significant increase in FLI in the middle section of the DMPAG following I-HEM but not HYDRAZ. Since activation of the DMPAG induces sympathoexcitation (6) and the I-HEM group was the only group that showed a significant increase in HR during the compensatory phase of HEM, this raises the possibility that activation of DMPAG area neurons plays an important role in maintaining MAP during the initial compensatory phase of hemorrhage. Accordingly, an increase in FLI was also reported, but not quantified, the middle region of the DMPAG by Keay et al. in response to 15% TBV withdrawal in conscious rats (22). On the other hand, it should be noted that chemical blockade of the dorsal PAG has been reported to have no effect on the cardiovascular response to severe HEM (7). Yet, in that study severe hemorrhage was induced 4-6 hours after isofluorane anesthesia and no compensatory change in HR was noted in the control conditions. Thus, it remains to be determined what role dorsal PAG neurons play in modulating cardiovascular responses to I-HEM in an anesthesia-free animal.
In the present study, increased levels of FLI following I-HEM compared to control were observed in all three rostral-caudal regions of the LPBN, including KF. Yet, only in the rostral LPBN was the increase in FLI during HEM separated from the effects of HYDRAZ or hypotension alone. More specifically, in the central lateral subnucleus of the rostral LPBN both I-HEM and F-HEM selectively induced increased levels of FLI. In contrast, the effect of HYDRAZ was not significantly different from SAL controls. These results compliment recent observations by Blair et al. (3) that smaller lesions of the LPBN, which encompassed only the dorsolateral portion, attenuated the bradycardic response to severe hemorrhage in conscious rats. In contrast, larger lesions involving the entire PBN (dorsolateral and ventrolateral subnuclei, possibly including KF) had little effect on the decompensatory response to severe hemorrhage but impaired recovery. This raises the possibility that that the central lateral subnucleus of the rostral LPBN may be involved in mediating or relaying signals associated with HIS. Indeed, this region of the rostral LPBN receives a large projection from the VLPAG (24), though the physiological function of this interconnection has yet to be determined.

Several regions outside of the PAG and PBN were also quantified for FLI levels in the present study. These regions included LC, the CnF nucleus, and A7. All three regions have been shown to be involved in responses to different types of stress (23, 43) and cardiovascular regulation (16, 31). In the present study all three regions demonstrated significant increases in FLI in response to I-HEM but not HYDRAZ. Since all three regions are interconnected with the PAG (1, 43) all three may be well positioned to contribute to cardiovascular adjustments during the transition from compensation to decompensation during hemorrhage. Future studies should focus on determining the role of each region in HIS.
In summary, the results of the present study provide a comprehensive look at the impact of different rates of severe hemorrhage on the cardiovascular outcome coupled with regional CNS activation. We have confirmed previous observations that suggested there are marked differences in autonomic regulation when severe hemorrhage occurs at different rates (42). The results of our study suggest that a constant withdrawal rate of 1 ml/kg/min until 30% TBV has been removed produces the most reliable pattern of tachycardia and compensation followed by hypotension and bradycardia for the study of experimental severe hemorrhage in conscious rats. Associated with this rate of hemorrhage were indicators of increased levels of excitation localized to the caudal VLPAG, the middle DMPAG, the rostral central lateral subnucleus of the LPBN, and LC. Other brain regions newly identified to be potentially involved in mediating hemorrhage responses include cuneiform nucleus and the A7 region. Together these results provide further evidence of the potential importance of activation of the rostral brainstem in mediating the response to severe hemorrhage.
TABLE 1. Baseline MAP and HR of treatment groups.

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<thead>
<tr>
<th>Treatment Group</th>
<th>MAP</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALINE (n=5)</td>
<td>109±4</td>
<td>351±14</td>
</tr>
<tr>
<td>HYDRAZ (n=5)</td>
<td>122±4</td>
<td>349±24</td>
</tr>
<tr>
<td>S-HEM (n=7)</td>
<td>120±4</td>
<td>353±5</td>
</tr>
<tr>
<td>I-HEM (n=7)</td>
<td>123±5</td>
<td>356±13</td>
</tr>
<tr>
<td>F-HEM (n=6)</td>
<td>123±4</td>
<td>366±23</td>
</tr>
</tbody>
</table>

No significant different between groups ($P>0.05$).
TABLE 2. Cardiovascular data averaged at the time of the offset of HEM compared to HYDRAZ at the corresponding time point.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>TIME POINT (min.)</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-HEM (n=7)</td>
<td>40</td>
<td>76±6</td>
<td>294±23*</td>
</tr>
<tr>
<td>HYDRAZ (n=5)</td>
<td></td>
<td>80±3</td>
<td>480±11</td>
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<tr>
<td>I-HEM (n=7)</td>
<td>20</td>
<td>73±15</td>
<td>277±34*</td>
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<tr>
<td>HYDRAZ (n=5)</td>
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<td>84±5</td>
<td>487±14</td>
</tr>
<tr>
<td>F-HEM (n=6)</td>
<td>10</td>
<td>68±6*</td>
<td>305±35*</td>
</tr>
<tr>
<td>HYDRAZ (n=5)</td>
<td></td>
<td>89±5</td>
<td>481±20</td>
</tr>
</tbody>
</table>

* Indicates significant difference from HYDRAZ average ($P<0.01$).
FIGURE LEGENDS

Figure 1. Schematic of periaqueductal grey (PAG) and lateral parabrachial nucleus (LPBN) areas imaged for quantification of Fos-positive staining. All figures and numbers were adapted from Paxinos and Watson (33). Approximate middle, and caudal PAG (A) and rostral, middle, and caudal LPBN (B) areas used. Numbers displayed with each representative section indicate approximate coordinates caudal to Bregma. DMPAG, dorsal medial periaqueductal grey; VLPAG, ventrolateral periaqueductal grey; Sup, superior lateral parabrachial; Ctr, central lateral parabrachial; KF, Kolliker-Fuse nucleus; SCP, superior cerebellar peduncle; Dor, dorsal lateral parabrachial; Cres, crescent lateral parabrachial; Ext, external lateral parabrachial.

Figure 2. MAP (A) and HR (B) responses to saline (SAL; n = 5) or hydralazine (HYDRAZ; n = 5) infusion. Minute 0 represents baseline. * indicates significantly different (P<0.05) from baseline within treatment group. # indicates significantly different (P<0.05) from HYDRAZ treated group at specific time point.

Figure 3. MAP (A) and HR (B) responses to slow (0.5 ml/kg/min; S-HEM; n=7), intermediate (1.0 ml/kg/min; I-HEM, n=7), and fast (2.0 ml/kg/min; F-HEM, n=6) rates of hemorrhage. Minute 0 represents baseline. The lowest MAP for each rate of HEM corresponds with the point at which 30% of total blood volume had been removed (i.e. cessation of blood withdrawal). @ -indicates significantly different (P<0.05) from baseline for the F-HEM group. * indicates significantly different from baseline for the I-HEM group. $ indicates significantly
different from baseline for the S-HEM group. # indicates F-HEM value was significantly different from both I-HEM and S-HEM at specified time point. δ indicates S-HEM value was significantly different from both I-HEM and F-HEM at specified time point.

Figure 4. Representative images of Fos-like immunoreactive neurons in the middle and caudal periaqueductal gray (PAG) of two individual animals that underwent either saline injection (SAL) or severe hemorrhage at an intermediate (I-HEM) rate. Aq, central aqueduct; DMPAG, dorsal medial PAG; DLPAG, dorsal lateral PAG; LPAG, lateral PAG; VLPAG, ventrolateral PAG. Numbers in the lower left of the ventral sections of SAL animal indicate approximate location of representative sections relative to bregma in mm (33).

Figure 5. Average Fos-positive cell counts from the middle and caudal DMPAG (A &B) and VLPAG (C& D) following different rates of severe hemorrhage and hydrazaline-induced hypotension. M = middle PAG (defined as -7.5 to -7.8 mm caudal from bregma); C = caudal PAG (defined as -8.0 to -8.4 mm caudal from bregma) (33) . SAL = saline control (n=5); S-HEM = slow rate of 30% hemorrhage (n=7); I-HEM = intermediate rate of 30% hemorrhage (n=7); F-HEM = fast rate of 30% hemorrhage (n=6); HYDRAZ = hydrazaline treated animals (n=5). * indicates significantly different from SAL.

Figure 6. Representative images of Fos-like immunoreactive neurons in the rostral, middle, and caudal lateral parabrachial nucleus (LPBN) of rats that underwent either saline injection (SAL) or severe hemorrhage at a fast (F-HEM) or intermediate (I-HEM) rate. Subnuclei of the LPBN outlined include: Sup=superior; Ctr=central; Dor=dorsal; Cres,
lateral crescent; Ext, external. SCP is the superior cerebellar peduncle. Numbers in the lower left of SAL animal indicate approximate location of representative sections relative to bregma in mm (33).

Figure 7. Average Fos-positive cell counts from selected subnuclei the rostral (A) middle (B) and caudal (C) LPBN following different rates of severe hemorrhage and hydrazaline-induced hypotension. Rostral LPBN was defined as -8.8 to -9.0 mm caudal from bregma. Middle LPBN was defined as -9.05 to 9.3 mm caudal to bregma. Caudal LPBN was defined as -9.35 to -9.6 mm caudal from bregma (33). SAL = saline control (n=5); S-HEM = slow rate of 30% hemorrhage (n=7); I-HEM = intermediate rate of 30% hemorrhage (n=7); F-HEM = fast rate of 30% hemorrhage (n=6); HYDRAZ = hydrazaline treated animals (n=5). * indicates significantly different from SAL. Horizontal bars indicate that only the group of subnuclei, not individual subnuclei, was significantly different from SAL. Note the difference in y-axis scales.

Figure 8. Fos-positive cells quantified in the Cuneiform Nucleus (A), Locus Coeruleus (B), Kolliker-Fuse Nucleus (C), and A7 cell group (D) following different rates of severe hemorrhage and hydrazaline-induced hypotension. SAL = saline control (n=5); S-HEM = slow rate of 30% hemorrhage (n=7); I-HEM = intermediate rate of 30% hemorrhage (n=7); F-HEM = fast rate of 30% hemorrhage (n=6); HYDRAZ = hydrazaline treated animals (n=5). * indicates significantly different from SAL.
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